APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: OCTAPEPTIDE BOMBESIN ANALOGS

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OCTAPEPTIDE BOMBESIN ANALOGS
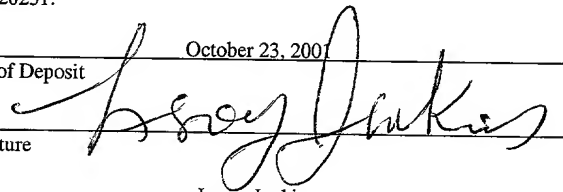
Background of the Invention

This application is a continuation-in-part of
U.S. Patent Application Serial No. 397,169, filed August
21, 1989, which is a continuation-in-part of U.S. Patent
Application Serial No. 376,555, filed July 7, 1989,
which is a continuation-in-part of U.S. Patent
Application Serial No. 317,941, filed March 2, 1989,
which is a continuation-in-part of U.S. Patent
Application Serial Number 282,328, filed December 9,
1988, which in turn is a continuation-in-part of U.S.
Patent Application Serial No. 257,998, filed October 14,
1988, which in turn is a continuation-in-part of U.S.
Patent Application Serial No. 248,771, filed September
23, 1988, which in turn is a continuation-in-part of Coy
et al., U.S. Patent Application Serial No. 207,759,
filed June 16, 1988, which in turn is a
continuation-in-part of Coy et al., U.S. Patent
Application Serial No. 204,171, filed June 8, 1988,
which in turn is a continuation-in-part of Coy et al.,
U.S. Patent Application Serial No. 173,311, filed March
25, 1988, which in turn is a continuation-in-part of Coy
et al. U.S. Patent Application Serial No. 100,571, filed
September 24, 1987.

This invention relates to therapeutic peptides
useful, e.g., for treatment of benign or malignant
proliferation of tissue, for gastrointestinal disorders,
and for diabetes.

The amphibian peptide bombesin, pGlu-Gln-Arg- SEQ ID No. 1
Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$
(Anastasi et al., Experientia $\underline{27}$:166-167 (1971)), is
closely related to the mammalian gastrin-releasing
peptides (GRP), e.g., the porcine GRP, H$_2$N-

Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-
Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-   SEQ ID # 2
Val-Gly-His-Leu-Met-(NH_2) (McDonald et al., Biochem.
Biophys. Res. Commun. 90:227-233 (1979)) and human GRP,
H_2N-Val-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-Leu-Thr-Lys-   SEQ ID # 3
Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met
(NH_2). Bombesin has been found to be a growth factor
for a number of human cancer cell lines, including
small-cell lung carcinoma (SCLC), and has been detected
in human breast and prostate cancer (Haveman et al.,
eds. Recent Results in Cancer Research - Peptide
Hormones in Lung Cancer, Springer-Verlag, New
York:1986). A number of these cancers are known to
secrete peptide hormones related to GRP or bombesin.
Consequently, antagonists to bombesin have been proposed
as agents for the treatment of these cancers.

        Cuttitta et al. demonstrated that a specific
monoclonal antibody to bombesin inhibited in vivo the
growth of a human small-cell lung cancer cell line
xenografted to nude mice (Cuttitta et al., Cancer Survey
4:707-727 (1985)). In 3T3 murine fibroblasts which are
responsive to the mitotic effect of bombesin, Zachary
and Rozengurt observed that a substance P antagonist
(Spantide) acted as a bombesin antagonist (Zachary et
al., Proc. Natl. Acad. Sci. (USA), 82:7616-7620
(1985)). Heinz-Erian et al. replaced His at position 12
in bombesin with D-Phe and observed bombesin antagonist
activity in dispersed acini from guinea pig pancreas
(Heinz-Erian et al., Am. J. of Physiol. 252:G439-G442
(1987)). Rivier reported work directed toward
restricting the conformational freedom of the bioactive
C-terminal decapeptide of bombesin by incorporating
intramolecular disulfide bridges; however, Rivier

mentioned that, so far, bombesin analogs with this
modification fail to exhibit any antagonist activity
(Rivier et al., "Competitive Antagonists of Peptide
Hormones," in Abstracts of the International Symposium
on Bombesin-Like Peptides in Health and Disease, Rome,
Italy (October, 1987).

Abbreviations (uncommon):

cyclohexyl-Ala =   (cyclohexyl alanine)

$$H_2C \begin{matrix} H_2 \\ C \end{matrix}$$

identifying group

$$NH_2-CH-CO_2H;$$
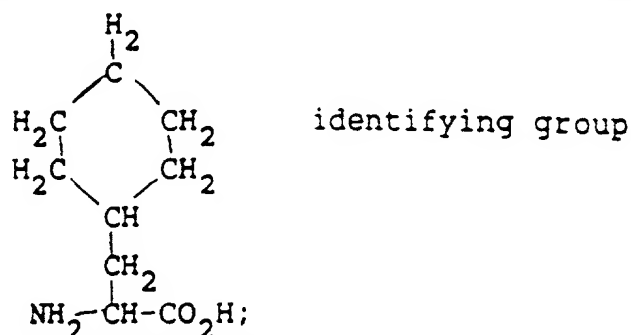
pGlu = H_2C--CH-COOH (pyroglutamic acid);
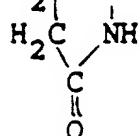
Nle = $H_2N-CH-COOH$ (norleucine)
       $(CH_2)_3-CH_3$
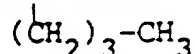
Pal = 3-pyridyl-alanine

ß-leu = ß - homoleucine

γ-leu = gamma - homoleucine

D-Cpa = D-p-chlorophenylalanine

HyPro = hydroxyproline

Nal   = naphthylalanine

Sar   = sarcosine

F_5-Phe   = penta-fluoro-Phenylalanine

Sta (statine) =

     (3S, 4S)-4-amino-3-hydroxy-6-methylheptanoic acid,
and has the chemical structure:

$$CH_3-CH(CH_3)-CH_2$$

identifying group

$$NH_2-CH-CH-CH_2-CO_2H;$$
$$\qquad\quad |$$
$$\qquad\quad OH$$

AHPPA =

(3S,4S)-4-amino-3-hydroxy-5-phenylpentanoic acid, and has the chemical structure:

identifying group

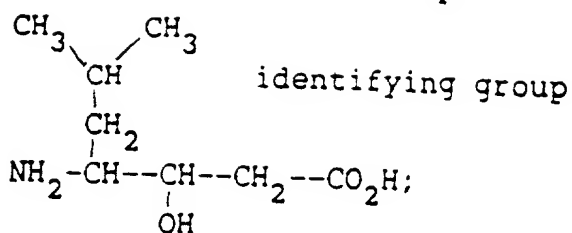$$NH_2-CH-CH-CH_2-\overset{O}{\overset{\|}{C}}-OH$$
$$\qquad\quad |$$
$$\qquad\quad OH$$
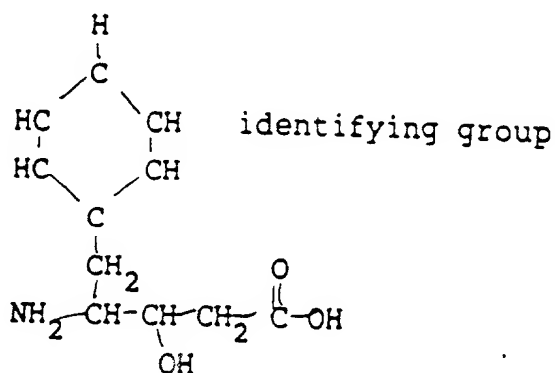
ACHPA =

(3S, 4S)-4-amino-5-cyclohexyl-3-hydroxypentanoic acid and has the chemical structure:

identifying group

$$NH_2-CH-CH-CH_2-\overset{O}{\overset{\|}{C}}-OH;$$
$$\qquad\quad |$$
$$\qquad\quad OH$$
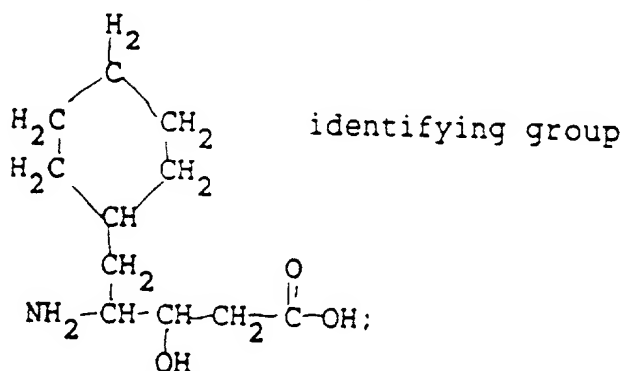
R = right (D) configuration; S = left (L) configuration; racemate = equal mix of R and S

1-methyl-His; 3-methyl-His = methyl (CH_3) group on nitrogen at positions 1 or 3 of Histidine:

$$NH_2--CH--COOH$$
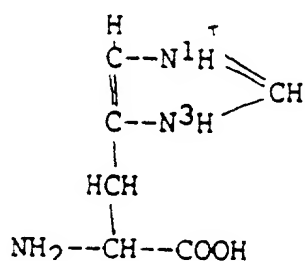
Summary of the Invention

In general, the invention features a linear
(i.e., non-cyclic) analog of biologically active
mammalian gastrin-releasing peptide (GRP), amphibian
bombesin, or mammalian growth hormone releasing factor
(GRF) having an active site and a binding site
responsible for the binding of the peptide to a receptor
on a target cell; cleavage of a peptide bond in the
active site of naturally occurring bombesin, GRP, or GRF
is unnecessary for in vivo biological activity. The
analog has one of the following modifications: (a) a
deletion of an amino acid residue within the active site
and a modification of an amino acid residue outside of
the active site, (b) a replacement of two amino acid
residues within the active site with a synthetic amino
acid, e.g., statine, AHPPA, or ACHPA, a ß-amino acid, or
a γ-amino acid residue, or (c) a non-peptide bond
instead of a peptide bond between an amino acid residue
of the active site and an adjacent amino acid residue.

In preferred embodiments the analog is capable
of acting as a competitive inhibitor of the naturally
occurring peptide by binding to the receptor and, by
virtue of one of the modifications, failing to exhibit
the in vivo biological activity of the naturally
occurring peptide.

The locations of the modifications that give
rise to antagonists are determined by the location of
the active site in the naturally occuring peptide. For
example, the linear peptides for which introduction of a
non-peptide bond between the carboxyl terminal and
adjacent amino acid residues, or the replacement of the
natural carboxyl terminal and adjacent amino acid
residues with a synthetic, ß-, or γ- amino acid

residue, or the deletion ("des") of the C-terminal amino acid residue are useful in creating or enhancing antagonist activity are those in which activity is associated with the two C-terminal amino acid residues of the amino acid chain. Similarly, where the active site is located in the amino terminal portion of the naturally occuring peptide, the corresponding analogs of the invention will possess modifications in their amino terminal portions.

In preferred embodiments the active site includes at least one amino acid residue located in the carboxyl terminal half of the naturally occurring biologically active peptide and that amino acid residue is located in the carboxyl terminal half of the linear peptide.

In preferred embodiments the binding sites includes at least one amino acid residue located in the amino terminal half of the naturally occurring biologically active peptide and that amino acid residue is located in the amino terminal half of the linear peptide.

Modifications can be introduced in a region involved in receptor binding, or in a non-binding region. Preferably, analogs of the invention are 25% homologous, most preferably, 50% homologous, with the naturally occurring peptides.

The analogs of the invention may have one of the modifications given in the generic formula given below either a non-peptide bond instead of a peptide bond between an amino acid residue of the active site and an adjacent amino acid residue; or a synthetic amino acid, e.g. a statine, an AHPPA, or an ACHPA, a ß-amino acid, or a γ-amino acid residue in place of two

natural amino acid residues; or a deletion of the
C-terminal amino acid residue, accompanied by the
addition of a substituent on the actual C-terminal group
and the presence of an N-terminal residue that is not
the natural N-terminal amino acid residue of the
peptides from which the analogs are derived. (Statine,
AHPPA, and ACHPA have the chemical structures defined
above. Where statine is used herein, AHPPA or ACHPA may
also be used.)

By non-peptide bond is meant that the carbon
atom participating in the bond between two residues is
reduced from a carbonyl carbon to a methylene carbon,
i.e., CH_2-NH; or, less preferably that CO-NH is
replaced with any of CH_2-S, CH_2-O, CH_2-CH_2,
CH_2-CO, or CO-CH_2. (A detailed discussion of the
chemistry of non-peptide bonds is given in Coy et al.
(1988) Tetrahedron 44,3:835-841, hereby incorporated by
reference, Tourwe (1985) Janssen Chim. Acta 3:3-15,
17-18, hereby incorporated by reference, and Spatola
(1983) in Chemistry and Biochemistry of Amino Acids,
Peptides, and Proteins, (B. Weinstein, ed.) M. Dekker,
New York and Basel, pp. 267-357, hereby incorporated by
reference.)

One modification of the naturally occurring
peptide to create an antagonist is of the amino terminal
end of the molecule, such as those described for the
amino terminal positions in the generic formula below;
for example, the N-terminal amino acid residue, which is
A^0 or, if A^0 is deleted, is A^1 or, if A^0 and
A^1 are deleted, is A^2 below, may be an aromatic
D-isomer, or may be an alkylated amino acid residue.
(Where "D" is not designated as the configuration of an
amino acid, L is intended.)

The therapeutic peptide includes between seven and ten amino acid residues, inclusive, and is an analog of one of the following peptides terminating at the carboxy-terminus with a Met residue: (a) litorin; (b) the ten amino acid carboxy-terminal region of mammalian GRP; and (c) the ten amino acid carboxy-terminal region of amphibian bombesin. The therapeutic peptide is of the following formula:

$$\begin{matrix} R_1 \\ \diagdown \\ \diagup \\ R_2 \end{matrix} A^0 - A^1 - A^2 - Trp - A^4 - A^5 - A^6 - A^7 - W$$
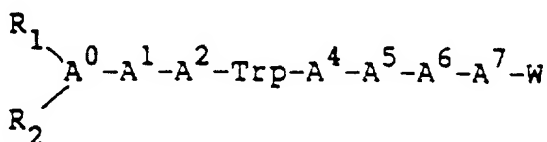
wherein

A^0 = pGlu, Gly, Nle, α-aminobutyric acid, or the D-isomer of any of Ala, Val, Gln, Asn, Leu, Ile, Met, p-X-Phe (where X = F, Cl, Br, NO_2, OH, H or CH_3), Trp, Cys, or ß-Nal, or is deleted; -

A^1 = the D or L-isomer of any of pGlu, Nle, α-aminobutyric acid, or the D-isomer of any of Ala, Val, Gln, Asn, Leu, Ile, Met, p-X-Phe (where X = F, Cl, Br, NO_2, OH, H or CH_3), F_5-Phe, Trp, Cys, or ß-Nal, or is deleted;

A^2 = pGlu, Gly, Ala, Val, Gln, Asn, Leu, Ile, Met, p-X-Phe (where X = F, Cl, Br, NO_2, OH, H or CH_3), Trp, Cys, ß-Nal, His, 1-methyl-His, or 3-methyl-His;

A^4 = Ala, Val, Gln, Asn, Gly, Leu, Ile, Nle, α-aminobutyric acid, Met, p-X-Phe (where X = F, Cl, Br, NO_2, OH, H or CH_3), Trp, Cys, or ß-Nal;

A^5 = Gln, Asn, Gly, Ala, Leu, Ile, Nle, α-aminobutyric acid, Met, Val, p-X-Phe (where X = F, Cl, Br, OH, H or CH_3), Trp, Thr, or ß-Nal;

A^6 = Sar, Gly, or the D-isomer of any of Ala, N-methyl-Ala, Val, Gln, Asn, Leu, Ile, Met, p-X-Phe (where X = F, Cl, Br, NO_2, OH, H or CH_3), Trp, Cys, or ß-Nal;

A^7 = 1-methyl-His, 3-methyl-His, or His;

provided that, if A^0 is present, A^1 cannot be pGlu; further provided that, if A^0 or A^1 is present, A^2 cannot be pGlu; further provided that, when A^0 is deleted and A^1 is pGlu, R_1 must be H and R_2 must be the portion of Glu that forms the imine ring in pGlu; and further provided that, W can be any one of the following:

(I):

$$-NH-\overset{\overset{\displaystyle Z_1}{|}}{C}H-R_3-\overset{\overset{\displaystyle O}{\|}}{C}-V,$$
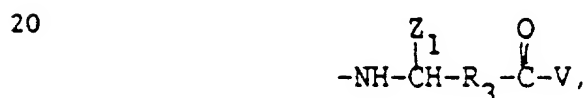
wherein R_3 is $CHR_{20}-(CH_2)_{n1}$ (where R_{20} is either of H or OH; and n1 is either of 1 or 0), or is deleted, and Z_1 is the identifying group of any of the amino acids Gly, Ala, Val, Leu, Ile, Ser, Asp, Asn, Glu, Gln, p-X-Phe (where X = H, F, Cl, Br, NO_2, OH, or CH_3), F_5-Phe, Trp, Cys, Met, Pro, HyPro, cyclohexyl-Ala, or ß-nal; and V is either OR_4, or

$$N\overset{\displaystyle \diagup R_5}{\underset{\displaystyle \diagdown R_6}{}},$$
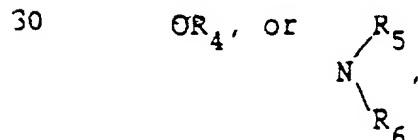
where R_4 is any of C_{1-20} alkyl, C_{3-20} alkenyl, C_{3-20} alkinyl, phenyl, naphthyl, or C_{7-10} phenylalkyl, and each R_5, and R_6, independently, is any of H, C_{1-12} alkyl, C_{7-10} phenylalkyl, lower acyl, or,

$$-N \begin{array}{c} R_{22} \\ \diagdown \\ H \end{array} ,$$
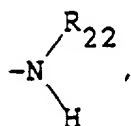
where R_{22} is any of H, C_{1-12} alkyl, C_{7-10} phenylalkyl, or lower acyl; provided that, when one of R_5 or R_6 is $-NHR_{22}$, the other is H;

(II):

$$\begin{array}{ccc} R_3 \, Z_1 & Z_2 \, O \\ | \;\; | & | \;\; || \\ N-CH-R_4-CH-C-V, \end{array}$$
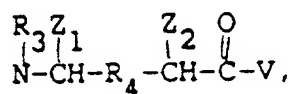
wherein R_4 is CH_2-NH, CH_2-S, CH_2-O, $CO-CH_2$, CH_2-CO, or CH_2-CH_2, and each Z_1 and Z_2, independently, is the identifying group of any one of the amino acids Gly, Ala, Val, Leu, Ile, Ser, Asp, Asn, Glu, Gln, ß-Nal, p-X-Phe (where X = H, F, Cl, Br, NO_2, OH or CH_3), F_5-Phe, Trp, Cys, Met, Pro, HyPro, or cylcohexyl-Ala; and V is either OR_5 or

$$N \begin{array}{c} \diagup R_6 \\ \diagdown R_7 \end{array}$$
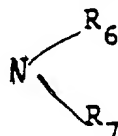
where each R_3, R_5, R_6, and R_7, independently, is H, lower alkyl, lower phenylalkyl, or lower naphthylalkyl;

(III):

$$
\begin{array}{c}
\quad\;\; Z_4\; Z_1 \;\; O \qquad Z_2 \\
\quad\quad |\quad |\quad\; || \qquad / \\
-N-CH-C-N \\
\qquad\qquad\qquad\quad \backslash \\
\qquad\qquad\qquad\qquad Z_3
\end{array}
,
$$
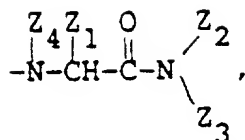
wherein Z_1 is the identifying group of any one of the amino acids Gly, Ala, Val, Leu, Ile, Ser, Asp, Asn, Glu, ß-Nal, Gln, p-X-Phe (where X = H, F, Cl, Br, NO_2, OH or CH_3), F_5-Phe, Trp, Cys, Met, Pro, or HyPro; and each Z_2, Z_3, and Z_4, independently, is H, lower alkyl, lower phenylalkyl, or lower naphthylalkyl; or

(IV):

$$
\begin{array}{c}
\qquad / Z_{20} \\
-N \\
\qquad \backslash Z_{30}
\end{array}
,
$$
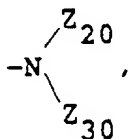
wherein each Z_{20} and Z_{30}, independently, is H, lower alkyl, lower phenylalkyl, lower naphthylalkyl; further provided that, when either of Z_{20} or Z_{30} is other than H, A^7 is His, A^6 is Gly, A^5 is Val, A^4 is Ala, A^2 is His, and either of R_1 or R_2 is other than H, A^1 must be other than deleted; further provided that, for the formulas (I) through (IV), any asymmetric carbon atom can be R, S or a racemic mixture; and further provided that each R_1 and R_2, independently, is H, C_{1-12} alkyl, C_{7-10} phenylalkyl, COE_1 (where E_1 is C_{1-20} alkyl, C_{3-20} alkenyl, C_{3-20} alkinyl, phenyl, naphthyl, or C_{7-10} phenylalkyl), or lower acyl, and R_1 and R_2 are

bonded to the N-terminal amino acid of said peptide, and further provided that when one of R_1 or R_2 is COE_1, the other must be H, or a pharmaceutically acceptable salt thereof.

Preferably, the amino acid sequence of the therapeutic peptide of the generic formula (id) is at least 25% homologous with the amino acid sequence of the naturally occurring peptide; most preferably, this homology is at least 50%.

More preferably, in the generic formula above,

A^0 = Gly, D-Phe, or is deleted;

A^1 = p-Glu, D-Phe, D-Ala, D-ß-Nal, D-Cpa, or D-Asn;

A^2 = Gln, His, 1-methyl-His, or 3-methyl-His;

A^4 = Ala;

A^5 = Val;

A^6 = Sar, Gly, D-Phe, or D-Ala;

A^7 = His; and

and, where W is (I) and R_3 is CH_2 or CH_2-CH_2, Z_1 is the identifying group of Leu or Phe, where W is (I) and R_3 is $CHOH$-CH_2, Z_1 is the identifying group of Leu, cyclohexyl-Ala, or Phe and each R_5 and R_6 is H, and where W is (I), V is NHR_6, and R_6 is NH_2; where W is (II) and R_4 is CH_2-NH each Z_1 is the identifying group of Leu, or Phe, and Z_2 is the identifying group of Leu or Phe; where W is (III), Z_1 is the identifying group of any one of the amino acids Leu or p-X-Phe (where X = H, F, Cl, Br, NO_2, OH or CH_3); and each Z_2, Z_3 and Z_4, independently, is H, lower alkyl, lower phenylalkyl, or lower naphthylalkyl; and where W is (IV), each Z_{20} and Z_{30}, is H; and each R_1 and R_2, independently, is H, lower alkyl, or lower acyl.

Preferred peptides include those in which W is (II),

R_4 is $CH_2 - NH$, and the carbon atom bonded to Z_2
is of the R configuration.

Examples of preferred bombesin or GRP peptides
are:

D-ß-Nal-Gln-Trp-Ala-Val-Gly-His-Leuψ[CH_2NH]Phe-NH_2,

D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-ethylamide,

p-Glu-Gln-Trp-Ala-Val-Gly-His-statine-amide, —— SEQ ID No

D-Phe-Gln-Trp-Ala-Val-Gly-His-Leuψ[CH_2NH]-D-Phe-NH_2,

D-Cpa-Gln-Trp-Ala-Val-Gly-His-ß-Leu-NH_2,

D-Cpa-Gln-Trp-Ala-Val-D-Ala-His-ß-Leu-NH_2,

D-Cpa-Gln-Trp-Ala-Val-Gly-His-Leuψ[CH_2NH]-Phe-NH_2.

In another preferred embodiment, W is (I), V is
OR_4, R_4 can be any substituent provided in (I) of
the generic formula (id), and A^6 is N-methyl-D-Ala,
i.e., a preferred peptide contains both a carboxy
terminal ester component in combination with
N-methyl-D-Ala, in position A^6; or $D-F_5$-Phe in
position A^1.

Examples of preferred peptides are

D-Phe-Gln-Trp-Ala-Val-N-methyl-D-Ala-His-Leu-methylester.

$D-F_5$-Phe-Gln-Trp-Ala-Val-D-Ala-His-Leu-methylester.

An example of a preferred GRF peptide of the
invention is

Tyr-Ala^2-Asp-Ala-Ile-Phe-Thr-Asn-Serψ[CH_2NH]Tyr-
Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-
Asp-Ile-Met-Ser-Arg-NH_2; most preferably, the peptide
contains, D-Ala, N-methyl-D-Ala, or a-aminobutyric acid
in position 2. (Non-peptide bonds in which the peptide
bond is reduced are symbolized herein by " ψ[CH_2NH]"
or "ψ".)

Antagonists of the invention are useful for
treating diseases involving the malignant or benign
proliferation of tissue, such as all forms of cancer

where bombesin-related, GRP-related, or GRF-related
substances act as autocrine or paracrine mitotic
factors, e.g., cancers of the gastrointestinal tract,
pancreatic cancer, colon cancer, lung cancer,
particularly the small cell subtype, prostate or breast
cancer; or for treating artherosclerosis, and disorders
of gastrointestinal tissues related to gastric and
pancreatic secretions and motility; for example, for
causing the suppression of amylase secretion, or for
appetite control. GRF antagonists suppress the release
of growth hormone and, therefore, may be used to slow
down the progression of muscular dystrophy or for
treating diabetes, or diabetes-related retinopathy.

In the generic formulas given above, when R_1,
R_2, R_4 of I, R_5 of I, R_6 of I, R_{22} of I, R_3
of II, R_5 of II, R_6 of II, R_7 of II, Z_2 of III,
Z_3 of III, Z_4 of III, Z_{20} of IV, or Z_{30} of IV is
an aromatic, lipophilic group, the _in vivo_ activity can
be long lasting, and delivery of the compounds of the
invention to the target tissue can be facilitated.

The identifying group of an α-amino acid is
the atom or group of atoms, other than the α-carbonyl
carbon atom, the α-amino nitrogen atom, or the H atom,
bound to the asymmetric α-carbon atom. To illustrate
by examples, the identifying group of alanine is CH_3,
the identifying group of valine is $(CH_3)_2CH$, the
identifying group of lysine is $H_3N^+(CH_2)_4$ and
the identifying group of phenylalanine is
$(C_6H_6)CH_2$. The identifying group of a β- or
γ-amino acid is the analagous atom or group of atoms
bound to respectively, the ß- or the γ-carbon atom.
Where the identifying group of an amino acid is not
specified it may be α, ß, or γ.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

We first briefly describe the drawings.

Drawings

Fig. 1 is a graph of tumor growth curves for the small cell lung cancer (NCI-H69) xenografts.

Fig. 2 is a series of amino acid sequences of naturally occurring peptides of which peptides of the invention are analogs.

Fig. 3 is a series of amino acid sequence of naturally occurring peptides of the VIP peptide family, of which GRF peptides of the invention are analogs.

Figs. 4 and 5 are graphs showing the effect of pseudopeptide bond containing analogs of $GRF(1-29)NH_2$ on GH secretion from dispersed rat pituitary cells.

Fig. 6 is a graph showing the antagonism of GRF stimulated GH secretion by $Ser^9\psi[CH_2NH]Tyr^{10}GRF(1-29)NH_2$. We now describe the structure, synthesis, and use of the preferred embodiments of the invention.

Structure

Peptides of the invention have either a non-peptide bond in at least one of the indicated positions, or a statine, ß-amino acid, or γ-amino acid substitution, e.g., sta^8-des-Met^9 litorin. By non-peptide bond is meant e.g., that the carbon atom participating in the bond between two residues is reduced from a carbonyl carbon to a methylene carbon. The peptide bond reduction method which yields this non-peptide bond is described in Coy et al., U.S. patent application, Serial No. 879,348, assigned to the same assignee as the

present application, hereby incorporated by reference.
Any one of the amino acids in positions 0, 1, 2, and 9
of the litorin antagonists may be deleted from the
peptides, and the peptides are still active as
antagonists.

The peptides of the invention can be provided
in the form of pharmaceutically acceptable salts.
Examples of preferred salts are those with
therapeutically acceptable organic acids, e.g., acetic,
lactic, maleic, citric, malic, ascorbic, succinic,
benzoic, salicylic, methanesulfonic, toluenesulfonic, or
pamoic acid, as well as polymeric acids such as tannic
acid or carboxymethyl cellulose, and salts with
inorganic acids such as the hydrohalic acids, e.g.,
hydrochloric acid, sulfuric acid, or phosphoric acid.

Synthesis of Litorin, Bombesin, and GRF Analogs

The synthesis of the bombesin antagonist
pGlu-Gln-Trp-Ala-Val-Gly-His-Leuψ[CH$_2$NH]Leu-NH$_2$
follows. Other bombesin, GRP, or GRF antagonists can be
prepared by making appropriate modifications of the
following synthetic methods.

The first step is the preparation of the
intermediate
pGlu-Gln-Trp-Ala-Val-Gly-His(benzyloxycarbonyl)-Leuψ[CH$_2$NH]Leu-benzhydrylamine resin, as follows.

Benzhydrylamine-polystyrene resin (Vega
Biochemicals, Inc.) (0.97 g, 0.5 mmole) in the chloride
ion form is placed in the reaction vessel of a Beckman
990B peptide synthesizer programmed to perform the
following reaction cycle: (a) methylene chloride; (b)
33% trifluoroacetic acid (TFA) in methylene chloride (2
times for 1 and 25 min. each); (c) methylene chloride;
(d) ethanol; (e) methylene chloride; and (f) 10%
triethylamine in chloroform.

The neutralized resin is stirred with alpha-t-butoxycarbonyl(Boc)-leucine and diisopropylcarbodiimide (1.5 mmole each) in methylene chloride for 1 hour, and the resulting amino acid resin is then cycled through steps (a) to (f) in the above wash program. Boc-leucine aldehyde (1.25 mmoles), prepared by the method of Fehrentz and Castro, Synthesis, p. 676 (1983), is dissolved in 5 ml of dry dimethylformamide (DMF) and added to the resin TFA salt suspension followed by the addition of 100 mg (2 mmoles) of sodium cyanoborohydride (Sasaki and Coy, Peptides 8:119-121 (1987); Coy et al., id.). After stirring for 1 hour, the resin mixture is found to be negative to ninhydrin reaction (1 min.), indicating complete derivatization of the free amino group.

The following amino acids (1.5 mmole) are then coupled successively in the presence diisopropylcarbodiimide (1.5 mmole), and the resulting amino acid resin is cycled through washing/deblocking steps (a) to (f) in the same procedure as above: Boc-His(benzyloxycarbonyl), Boc-Gly (coupled as a 6 M excess of the p-nitrophenylester), Boc-Val, Boc-Ala, Boc-Trp, Boc-Gln (coupled as a 6 M excess of the p-nitrophenylester), and pGlu. The completed resin is then washed with methanol and air dried.

The resin described above (1.6 g, 0.5 mmole) is mixed with anisole (5 ml) and anhydrous hydrogen fluoride (35 ml) at 0°C and stirred for 45 min. Excess hydrogen fluoride is evaporated rapidly under a stream of dry nitrogen, and free peptide is precipitated and washed with ether. The crude peptide is dissolved in a minimum volume of 2 M acetic acid and eluted on a column (2.5 x 100 mm) of Sephadex G-25 (Pharmacia Fine

Chemicals, Inc.). Fractions containing a major component by uv absorption and thin layer chromatography (TLC) are then pooled, evaporated to a small volume and applied to a column (2.5 x 50 cm) of octadecylsilane-silica (Whatman LRP-1, 15-20 µm mesh size).

The peptide is eluted with a linear gradient of 0-30% acetonitrile in 0.1% trifluoroacetic acid in water. Fractions are examined by TLC and analytical high performance liquid chromatography (HPLC) and pooled to give maximum purity. Repeated lyophilization of the solution from water gives 60 mg of the product as a white, fluffy powder.

The product is found to be homogeneous by HPLC and TLC. Amino acid analysis of an acid hydrolysate confirms the composition of the peptide. The presence of the Leuψ[CH_2-NH]Leu bond is demonstrated by fast atom bombardment mass spectrometry.

pGlu-Gln-Trp-Ala-Val-Gly-His-Pheψ[CH_2NH]Leu-NH_2

and

pGlu-Gln-Trp-Ala-Val-Gly-His-Leuψ[CH_2NH]Leu-NH_2 or other peptides are prepared in similar yields in an analogous fashion by appropriately modifying the above procedure.

Solid phase synthesis of D-Phe[1], Leu[8]ψ[CH_2NH]D-Phe[9]-litorin, D-Phe-Gln-Trp-Ala-Val- Gly- His-Leuψ[CH_2NH]-D-Phe-NH_2, was carried out as follows:

Boc-D-Phe-Gln-Trp-Ala-Val-Gly-His(tosyl)-Leuψ[CH_2NH]-D-Phe-benzhydrylamine resin was synthesized first.

Benzhydrylamine-polystyrene resin (Advanced ChemTech, Inc.) (1.25 g, 0.5 mmole) in the chloride ion form is placed in the reaction vessel of an Advanced

ChemTech ACT 200 peptide synthesizer programmed to
perform the following reaction cycle: (a) methylene
chloride; (b) 33% trifluoroacetic acid in methylene
chloride (2 times for 1 and 25 min each); (c) methylene
chloride; (d) ethanol; (e) methylene chloride; (f) 10%
triethylamine in chloroform.

The neutralized resin is stirred with
Boc-D-phenylalanine and diisopropylcarbodiimide (1.5
mmole each) in methylene chloride for 1 h and the
resulting amino acid resin is then cycled through steps
(a) to (g) in the above wash program. The Boc group is
then removed by TFA treatment. Boc-leucine aldehyde
(1.25 mmoles), prepared by the method of Fehrentz and
Castro (1), is dissolved in 5 ml of dry DMF and added to
the resin TFA salt suspension followed by the addition
of 100 mg (2 mmoles) of sodium cyanoborohydride (2,3).
After stirring for 1 h, the resin mixure is found to be
negative to ninhydrin reaction (1 min) indicating
complete derivatization of the free amino group.

The following amino acids (1.5 mmole) are then
coupled successively by the same procedure:
Boc-His(benzyloxycarbonyl), Boc-Gly, Boc-Val, Boc-Ala,
Boc-Trp, Boc-Gln (coupled in the presence of 1 equiv.
hydroxybenzotriazole), Boc-D-Phe (coupled in the
presence of 1 equiv. hydroxybenzotriazole). After
drying, the peptide resin weighed 1.93 g.

The resin (1.93 g, 0.5 mmole) is mixed with
anisole (5 ml) and anhydrous hydrogen fluoride (35 ml)
at 0°C and stirred for 45 min. Excess hydrogen fluoride
is evaporated rapidly under a stream ot dry nitrogen and
free peptide precipitated and washed with ether. The
crude peptide is dissolved in a minimum volume of 2 M
acetic acid and eluted on a column (2.5 x 100 mm) of

Sephadex G-25. Fractions containing a major component
by uv absorption and thin layer chromatography are then
pooled, evaporated to a small volume and applied to a
column (2.5 x 50 cm) of Vydac octadecylsilane
(10-15 uM). This is eluted with a linear gradient of
15-45% acetonitrile in 0.1% trifluoroacetic acid in
water. Fractions are examined by thin layer
chromatography and analytical high performance liquid
chromatography and pooled to give maximum purity.
Repeated lyophlization of the solution from water gives
120 mg of the product as a white, fluffy powder.

The product is found to be homogeneous by hplc
and tlc. Amino acid analysis of an acid hydrolysate
confirms the composition of the octapeptide. The
presence of the Leuψ[CH$_2$NH] peptide bond is
demonstrated by fast atom bombardment mass
spectrometry.

Solid phase synthesis of
D-Phe^1-Leu^{8}des-Met^9 litorin, D-Phe-Gln-Trp-Ala-
Val-Gly-His-Leu-NH$_2$, was carried out as follows.

Step (1): Benzhydrylamine-polystyrene resin
(Advanced ChemTech, Inc. (0.62 gm, 0.25 mmole) in the
chloride ion form is placed in the reacton vessel of an
ACT 200 peptide synthesizer programmed to perform the
following reaction cycle: (a) methylene chloride; (b)
33% trifluoroacetic acid in methylene chloride (2 times
for 1 and 25 min each); (c) methylene chloride; (d)
ethanol; (e) methylene chloride; (f) 10% triethylamine
in chloroform.

The neutralized resin is stirred with
Boc-leucine and diisopropylcarbodiimide (1.5 mmole each)
in methylene chloride for 1 hr and the resulting amino
acid resin is then cycled through steps (a) to (g) in

the above wash program. The following amino acids (1.5
mmole) are then coupled successively by the same
procedure: Boc-His (benzyloxycarbonyl), Boc-Gly,
Boc-Val, Boc-Ala, Boc-Trp, Boc-Gln (coupled as a 6M
excess of the p-nitrophenylester, and pGlu (coupled in
the presence of hydroxzybenzotriazole). After drying,
the peptide resin weighed 0.92 g.

Step (2): The resin (0.92 g) is then mixed with
anisole (5 ml), dithiothreitol (200 mg) and anhydrous
hydrogen fluoride (35 ml) at 0° C and stirred for 45
min. Excess hydrogen fluoride is evaporated rapidly
under a stream of dry nitrogen and free peptide
precipitated and washed with ether. The crude peptide
is dissolved in a minimum volume of 2 M acetic acid and
eluted on a column (2.5 x 100 cm) of Sephadex G-25.
Fractions containing a major component by UV absorption
and thin layer chromatography are then pooled,
evaporated to a small volume and applied to a column
(2.5 x 50 cm) of Vydac octadecylsilane (10-15 microM).
The column is eluted with a linear gradient of 0-30%
acetonitrile in 0.1% trifluoroacetic acid in water.
Fractions are examined by thin layer chromatography and
pooled to give maximum purity. Repeated lyophilization
of the solution from water gives a white, fluffy powder;
this product is found to be homogeneous by hplc and
tlc. Amino acid analysis of an acid hydrolysate
confirms the composition of the peptide.

Synthesis of D-Nal-Gln-Trp-Ala-Val-Gly-
His-Leu-NH$_2$ was accomplished using the same procedure
as described above (0.62 g, 0.25 mmole of
benzyhydrylamine resin in step (1), and 0.92 g in step
(2)).

Synthesis of N-acetyl-D-Phe-Gln-Trp-Ala-Val-
Gly-His-Leu-NH$_2$ was accomplished using the same

procedure as that described above, using 0.62 g (0.25 mmole) of benzhydrylamine resin in step (1), and mixing 0.92 g of the resin with anisole in step(2), except that the final Boc group was removed and the resin acetylated with acetic anhydride in methylene chloride.

Synthesis of D-Phe-Gln-Trp-Ala-Val-N-Me-D-Ala-His(Tos)-Leu-O-resin is as follows:

Boc-Leu-O-Merrifield resin (1.0 g. 0.5 mmole) is placed in the reaction vessel of an Advanced ChemTech ACT 200 automatic peptide synthesizer programmed to perform the following reaction/wash cycle: (a) methylene chloride; (b) 33% trifluoroacetic acid in methylene chloride (2 times for 1 and 25 min. each); (c) propanol; (d) dimethylformamide; (e) 10% triethylamine in dimethylformamide; (f) dimethylformamide.

The neutralized resin is stirred with Boc-N^{im}-tosyl-histidine and diisopropylcarbodiimide (1.5 mmole each) in methylene chloride for 1 h. and the resulting amino acid resin is then cycled through steps (a) to (f) in the above wash program. The Boc group is then removed by TFA treatment. The following amino acids (1.5 mmole) are then coupled successively by the same procedure: Boc-N-Me-D-Ala (purchased from Bachem, Inc., CA), Boc-Val, Boc-Ala, Boc-Trp, Boc-Gln (coupled in the presence of 1 equiv. hydroxybenzotriazole), and Boc-D-Phe. After the last coupling was complete, the final Boc group was removed by TFA treatment as already described. After drying, the peptide resin weighed 1.28 g.

Synthesis of D-F$_5$-Phe-Gln-Trp-Ala-Val-D-Ala-His(Tos)-Leu-O-resin is as follows:

This analogue is prepared under the same conditions described above, except that Boc-D-Ala is used in place of N-Me-D-Ala and Boc-D-F$_5$Phe in place of D-Phe.

Synthesis of D-F$_5$Phe-Gln-Trp-Ala-Val-D-Ala-His-Leu-methyl ester is as follows.

This peptide is cleaved from the Merrifield resin described above under the same conditions, to give 198 mg of the product as a white, fluffy powder.

The product is found to be homogeneous by hplc and tlc. Amino acid analysis of an acid hydrolysate confirms the composition of the octapeptide and fast atom bombardment mass spectrometry gives the expected molecular weight for the peptide.

Synthesis of D-Phe-Gln-Trp-Ala-Val-N-Me-D-Ala-His-Leu-methyl ester is as follows:

The Merrifield resin described above (1.28 g, 0.5 mmole) is suspended in methanol containing 10% triethylamine and stirred at room temperature for 3 days. After filtration, the solution is evaporated to an oil which is dissolved in water and eluted on a column of Vydac octadecylsilane (10-15 µM) with a linear gradient of 10-40% acetonitrile in 0.1% trifluoroacetic acid in water. Fractions are examined by thin layer chromatography and analytical high performance liquid chromatography and pooled to give maximum purity. Repeated lyophilization of the solution from water gives 49 mg of the product as a white, fluffy powder.

The product is found to be homogeneous by hplc and tlc. Amino acid analysis of an acid hydrolysate confirms the composition of the octapeptide and fast atom bombardment mass spectrometry gives the expected molecular weight for the peptide.

The synthesis of Sta[8]-des-Met[9] litorin follows. A statine, AHPPA, or ACHPA residue can be substituted in place of any two amino acids of the peptide, where the peptide contains only peptide bonds. For example, sta[8]-des Met[9] litorin was prepared in an analagous fashion by first coupling statine to the resin and then proceeding with the addition of Boc-His(benzylocarbonyl). Statine or Boc-statine can be synthesized according to the method of Rich et al., 1978, J. Organic Chem. 43: 3624; and Rich et al., 1980, J. Med. Chem. 23: 27, and AHPPA and ACHPA can be synthesized according to the method of Hui et al., 1987, J. Med. Chem. 30: 1287; Schuda et al., 1988, J. Org. Chem. 53:873; and Rich et al., 1988, J. Org. Chem. 53:869.

Solid-phase synthesis of the peptide BIM-26120, pGlu-Gln-Trp-Ala-Val-Gly-His-Sta-NH$_2$, was accomplished through the use of the following procedures in which alpha-t-butoxycarbonyl statine (prepared by the procedure of Rich et al., J. Org. Chem. 1978, 43, 3624) is first coupled to methylbenzhydrylamine-polystyrene resin. After acetylation, the intermediate p-Glu-Gln-Gln-Trp-Ala-Val-Gly-His(benzyloxycarbonyl)-Sta-methylbenzhydrylamine resin is prepared. The synthetic procedure used for this preparation follows in detail:

1. Incorporation of alpha-t-butoxycarbonyl statine on methylbenzhydrylamine resin.

Methylbenzhydrylamine-polystyrene resin (Vega Biochemicals, Inc.) (1.0 g, 0.73 mmol) in the chloride ion form is placed in the reaction vessel of a Vega 250C Coupler peptide synthesizer. The synthesizer was programmed to perform the following reactions: (a) methylene chloride; (b) 10% triethylamine in chloroform; (c) methylene chloride; and (d) dimethylformamide.

The neutralized resin is mixed for 18 hours with the preformed active ester made from alpha-t-butoxycarbonyl statine (1.46 mmol), diisopropyl carbodiimide (2 mmol), and hydroxybenzotriazole hydrate (1.46 mmol in dimethylformamide at 0° C. for one hour. The resulting amino acid resin is washed on the synthesizer with dimethylformamide and then methylene chloride. The resin mixture at this point was found by the Kaiser ninhydrin test (5 minutes) to have an 84% level of statine incorporation on the resin.

Acetylation was performed by mixing the amino-acid resin for 15 minutes with N-acetyl imidazole (5 mmol) in methylene chloride. Derivatization to the 94-99% level of the free amino groups of the resin was indicated by the Kaiser ninhydrin test (5 minutes). The Boc-statine-resin is then washed with methylene chloride.

2. Couplings of the Remaining Amino Acids.

The peptide synthesizer is programmed to perform the following reaction cycle: (a) methylene chloride; (b) 33% trifluoroacetic acid (TFA) in methylene chloride (2 times for 5 and 25 min. each); (c) methylene chloride; (d) isopropyl alcohol; (e) 10% triethylamine in chloroform; and (f) methylene chloride.

The following amino acids (2.19 mmol) are then coupled successively by diisopropyl carbodiimide (4 mmol) alone or diisopropyl carbodiimide (4 mmol) plus

hydroxybenzotriazole hydrate (1.47 or 0.73 mmol) and the resulting peptide-resin is washed on the synthesizer with dimethylformamide and then methylene chloride, and then cycled through the washing and deblocking steps (a) to (f) in the procedure described above.

Boc-His (benzyloxycarbonyl) (coupled in the presence of 2 equivalents hydroxybenzotriazole); Boc-Gly; Boc-Val; Boc-Ala and Boc-Trp (coupled as the preformed hydroxybenzotriazole active esters made by reaction at 0° C for one hour with 1 equivalent hydroxybenzotriazole hydrate); Boc-Gln and pGlu (also coupled as the preformed active esters of hydroxybenzotriazole made by reaction at 0° C for one hour with 1 equivalent hydroxybenzotriazole hydrate). The completed peptide-resin is then washed with methanol and air dried.

The peptide-resin described above (1.60 g, 0.73 mmol) is mixed with anisole (2.5 mL), dithioerythreitol (50 mg), and anhydrous hydrogen fluoride (30 mL) at 0° C. for one hour. Excess hydrogen fluoride is evaporated rapidly under a stream of dry nitrogen, and the free peptide is precipitated and washed with ether. The crude peptide is dissolved in 100 mL of 1 M acetic acid and the solution is then evaporated under reduced pressure. The crude peptide is dissolved in a minimum volume of methanol/water 1/1 and triturated with 10 volumes of ethyl acetate.

The triturated peptide is applied to a column (9.4 mm I.D. x 50 cm) of octadecylsilane-silica (Whatman Partisil 10 ODS-2 M 9). The peptide is eluted with a linear gradient of 20-80% of 20/80 0.1% trifluoroacetic acid/acetonitrile in 0.1% trifluoroacetic acid in water. Fractions are examined by TLC and analytical

high performance liquid chromatography (HPLC) and pooled
to give maximum purity. Lyophilization of the solution
from water gives 77 mg of the product as a white fluffy
powder.

Other compounds including D-Cpa[1], ß-leu[8],
desMet[9] Litorin can be prepared as above and tested
for effectiveness as agonists or antagonists in the test
program described below.

GRF analogues were prepared by solid-phase
synthesis on methylbenzhydrylamine resin. The parent
GRF(1-29) peptidyl-resin was assembled on
4-methylbenzhydrylamine functionalized, 1% crosslinked
polystyrene resin (0.41 mequiv. g^{-1}), on 2 mmol scale
utilizing an Advanced ChemTech ACT 200 synthesizer,
using the following protocol: deblocking, 33% TFA (1
min, 25 min); DCM wash cycle; PrOH wash cycle;
neutralization, 10% DIEA (2 wash cycles); DMF wash
cycle; coupling of preformed HOBt esters (formed during
deprotection), 45 min in DMF, 15 min DMAP; PrOH wash
cycle; DCM wash cycle. Coupling reactions were
monitored qualitatively with the ninhydrin test (Kaiser
et al, Anal. Biochem. 1970, 34, 595). The peptidylresin
was divided into aliquots and the various analogues then
assembled on a 0.25 mmol scale. The reduced peptide
bonds were formed by the reductive alkylation of the
deprotected N^{α}-amino group with the appropriate
protected amino acid aldehyde (3.0 equiv.) in the
presence of $NaBH_3CN$ (10 equiv.) in DMF (25 mL)
containing 1% acetic acid at ambient temperature for 16
h.

The introduction of the reduced peptide bond
was accomplished by the reductive alkylation of the

resin-bound peptide amino terminus with a preformed
protected amino acid aldehyde (Sasaki et al., Peptides
1987, 8, 119; Sasaki et al, J. Med. Chem. 1987, 30,
1162). The protected amino acid aldehydes were prepared
in two steps using a modification of the method of
Fehrentz and Castro: (Fehrentz, Synthesis, 1983, 676)
the protected amino acids were converted to the
corresponding N,O-dimethylhydroxamates by reaction with
an excess of N,O-dimethylhydroxylamine hydrochloride
(1.1 equiv.) and dicyclohexylcarbodiimide (1.1 equiv.)
in dichloromethane containing an excess of
diisopropylethylamine (4 equiv.) at 0°C. The reaction
was allowed to warm up to ambient temperature over 16 h
with stirring. The crude N,O-dimethylhydroxamates were
isolated as oils after washing with 3 M HCl (3 x 30 mL),
3 M NaOH (3 x 30 mL), water (3 x 30 mL), drying over
$MgSO_4$ and evaporation to dryness at reduced pressure.
The N,O-hydroxamates were then reduced with $LiAlH_4$ in
tetrahydrofuran at 0°C. The reaction was followed by
TLC and the crude aldehydes isolated as oils which were
briefly stored at -20°C until used. The isosteres were
formed by the reductive alkylation of the preformed
protected amino-acid aldehyde (3 equiv.) with an excess
of $NaBH_3CN$ in acidified DMF. The progress of the
alkylation was monitored with the qualitative ninhydrin
test and most reactions produced a pink or red colour
which was taken as the end point. The method was shown
to be free of racemization in a model study (Coy et al,
Tetrahedron 1988, 3, 835). However, appreciable
racemization can occur if the aldehyde is stored for a
prolonged period before use. Peptide assembly was
completed using the same protocol as before. No attempt
was made to block any remaining primary amino groups or

to protect the secondary amino group formed during the
alkylation, since previous work has shown this moiety to
be unreactive during subsequent coupling reactions
(Sasaki, Peptides, supra; Hocart et al., J. Med. Chem.
1988, 31, 1820. The peptides were cleaved from the
resin support with simultaneous side-chain acidolysis
using anhydrous hydrogen fluoride containing anisole
(~30% v/v) and dithiothreitol (~0.6% w/v) as
scavengers for 1h at 0°C.

The peptides were then purified by gel
filtration and RP-HPLC to a final purity of >92.5% as
judged by analytical RP-HPLC. The crude peptides were
subjected initially to gel permeation chromatography on
Sephadex G50 (2.5 x 100 cm) with 2 M acetic acid
eluent. Final purification was effected by preparative
RP-HPLC on C_{18} bonded silica gel (Vydac C_{18},
10-15 μm, 1.0 x 45 cm) eluted with a linear
acetonitrile gradient with a constant concentration of
trifluoroacetic acid (0.1% v/v). The linear gradient
was generated using a Chromat-a-Trol Model II (Eldex
Laboratories Inc.) gradient maker. The separations were
monitored at 280 nm, by TLC on silica gel plates (Merck
F60) and analytical RP-HPLC. The fractions containing
the product were pooled, concentrated in vacuo and
subjected to filtration. Each peptide was obtained as a
fluffy white powder of constant weight by lyophilisation
from aqueous acetic acid. The purity of the final
peptides was assessed by RP-HPLC. Analytical RP-HPCL's
were recorded using a Vydac C_{18} support (4.6 x 250 mm,
5 μm, 300 ångstrom pore size, Liquid Separations
Group). A linear gradient from 30% to 60% acetonitrile
over 30 min with a constant concentration of
trifluoroacetic acid (0.1% v/v) was employed for all the

analyses at a flow rate of 1.5 mL min^{-1}. Column
eluent was monitored at 215 nm. The retention time and
purity of each peptide was assessed by the Rainin
Dynamax HPLC Method Manager.

Amino acid analyses of the peptides was done by
hydrolysis in vacuo (110°C; 20 h) in 4 M
methanesulphonic acid containing 0.2% 3-(2-aminoethyl)-
indole[2,23] (Pierce). Amino acid analyses were
performed on the hydrolysates using an LKB 4150
analyser, equipped with an Ultropac 11 column (6 x 273
mm) and a Shimadzu C-R3A recording integrator with
in-house software. The buffer sequence pH 3.20 (17.5
min), pH 4.25 (32 min), pH 10.00 (borate; 16 min) and
temperature sequence 50°C (5 min), 55°C (5 min), 58°C
(39.5 min), 65°C (7 min), 80°C (17 min) were used.
Standard retention times were as follows: His, 65.1;
Lys, 70.1; NH_3, 74.3; Arg, 77.2 min respectively. The
results are shown in Table 5.

FAB-MS was conducted by Oneida Research
Services, Inc., Whitesboro, NY using a Finnigan TSQ-70
equipped with an Ion Tech FAB gun at 6 kV with a primary
current of 0.2 mA whilst scanning from 2800 to 3500
amu. The samples were dissolved in a "Magic Bullet"
matrix and the results are given in Table 4. The
position of the pseudopeptide bond in each of the
synthesized peptides is shown in Table 6.

Solid phase synthesis of
Ser$^9\psi$[CH$_2$NH]Tyr^{10}GRF(1-29),
Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Serψ[CH$_2$NH]Tyr-   *SEQ ID No 5*
Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-
Asp-Ile-Met-Ser-Arg-NH$_2$, was carried out as follows.

Methylbenzhydrylamine resin (Advanced ChemTech,
Inc.) (0.62 g, 0.25 mmole) in the chloride ion form is

placed in the reaction vessel of an Advanced ChemTech ACT 200 peptide synthesizer programmed to perform the following reaction cycle: (a) 33% trifluoroacetic acid in methylene chloride (2 times for 1 and 25 min each); (b) DCM wash; (c) PrOH wash; (d) neutralization; (e) 10% diisopropyl ethylamine (DIEA) in chloroform (2 washes); (f) 0.75 mM DMF wash; (g) coupling of preformed activated amino acid (HOBT) esters, 45 min. in DMF; (h) 15 min. DMAP; (i) PrOH wash; (j) DCM wash. The pseudopeptide bond was incorporated by racemization-free reductive alkylation with a preformed protected amino-acid aldehyde in the presence of $NaCNBH_3$ in acidified DMF, as described by Coy et al., 1988, Tetrahedron 44:835 and Hocart et al., 1988, J. Med. Chem. 31:1820.

The resin bound peptide was elongated by repeating cycles (a-j) to give Boc-Tyr-Arg-Lys-Ala-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Als-Arg-Leu-methylbenzhydrylamine. *SEQ ID No. 6* The Boc group is then removed by TFA treatment. Boc-serine aldehyde (0.75 mmoles), prepared by the method of Fehrentz and Castro (1), is dissolved in 5 ml of dry DMF and added to the resin TFA salt suspension followed by the addition of 100 mg (2 mmoles) of sodium cyanoborohydride (2,3). After stirring for 1 h, the resin mixure is found to be negative to ninhydrin reaction (1 min) indicating complete derivatization of the free amino group.

The following amino acids (1.5 mmole) are then coupled successively by the same procedure. Boc-Asn, Boc-Thr (benzyl), Boc-Phe, Boc-Ile, Boc-Ala, Boc-Asp (o-cyclohexyl ester), Boc-Ala, Boc-Tyr

(dichlorobenzyl). After drying, the peptide resin weighed 1 g.

The aldehydes were prepared by reduction of the protected dimethylhydroxamates with $LiAlH_4$ (0.75 equiv.) for 180 min. at 0°C. The resin (1.0 g, 0.25 mmole) is mixed with anisole (5 ml), anhydrous hydrogen fluoride (35ml), and 100 mg dithiothretol at 0°C and stirred for 45 min. Excess hydrogen fluoride is evaporated rapidly under a stream of dry nitrogen and free peptide precipitated and washed with ether. The crude peptide is dissolved in a minimum volume of 2 M acetic acid and eluted on a column (2.5 x 100 mm) of Sephadex G-50. Fractions containing a major component by uv absorption and thin layer chromatography are then pooled, evaporated to a small volume and applied to a column (2.5 x 50 cm) of Vydac octadecylsilane (10-15 uM). This is eluted with a linear gradient of 0-50% acetonitrile in 0.1% trifluoroacetic acid in water. Fractions are examined by thin layer chromatography and analytical high performance liquid chromatography and pooled to give maximum purity. Repeated lyophilization of the solution from water gives 8.0 mg of the product as a white, fluffy powder.

The product is found to be homogeneous by hplc and tlc. Amino acid analysis of an acid hydrolysate confirms the composition of the octapeptide. The presence of the $Ser\psi[CH_2NH]Tyr$ psuedo peptide bond is demonstrated by fast atom bombardment mass spectrometry.

A statine, AHPPA, ACHPA, ß-amino acid, or Y-amino acid residue is added in the same way as is a natural α-amino acid residue, by coupling as a Boc derivative.

All but one of the peptides shown in Table 4 gave satisfactory amino acid analyses and the expected FAB-MS value within the error of the methods (see Table 4 and Table 5). The analogue

5   [Asn$^8\psi$[CH$_2$NH]-Ser^9]GRF(1-29)NH$_2$ could not be synthesised. The reaction of Boc-Asn(Xan)CHO with Ser(Bzl)~[resin] was very slow and no appreciable reaction was noted after several prolonged alkylations. This lack of reactivity may be due to the steric

10   hindrance of the bulky side-chain protecting groups since, in a series of Substance P analogues, Boc-Gln(Xan)CHO was used successfully (Qain et al., J. Biol. Chem. 1989, 16667). No problems were experienced with the preparation or used of Boc-Asp(OCHx)CHO and no

15   evidence of over reduction to homo-Ser was observed.

Phase 1 - 3T3 Peptide Stimulated [^{3}H] Thymidine
                Uptake Assay

Cell Culture. Stock cultures of Swiss 3T3 cells are grown in Dulbecco's Modified Eagles Medium

20   (DMEM) supplemented with 10% fetal calf serum in humidified atmosphere of 10% CO$_2$/90% air at 37°C. For experimental use, the cells are seeded into 24-well cluster trays and used four days after the last change of medium. The cells are arrested in the G1/G0 phase of

25   the cell cycle by changing to serum-free DMEM 24 hours prior to the thymidine uptake assay.

Assay of DNA Synthesis. The cells are washed twice with 1ml aliquots of DMEM (-serum) then incubated with DMEM (-serum), 0.5μM [methyl-^{3}H] thymidine

30   (20Ci/mmole, New England Nuclear), bombesin (3nM), and initially four concentrations of the test compounds (1, 10, 100, 1000nM) in a final volume of 1.0 ml. After 28 hours at 37°C, [methyl-^{3}H] thymidine incorporation

into acid-insoluble pools is assayed as follows. The cells are washed twice with ice-cold 0.9% NaCl (1ml aliquots), and acid soluale radioactivity is removed by a 30 min. (4°C) incubation with 5% trichloroacetic acid (TCA). The cultures are then washed once (1ml) with 95% ethanol and solubilized by a 30 min. incubation (1ml) with 0.1N NaOH. The solubilized material is transferred to vials containing 10ml ScintA (Packard), and the radioactivity is determined by liquid scintillation spectrometry.

Phase 2 - Small Cell Carcinoma (SCLC) - Bombesin
        Stimulated [³H] Thymidine Uptake Assay

        Cell Culture. Cultures of the human cell carcinoma cell line (NCI-H69) (obtained from the American Type Culture Association) are maintained in RPMI 1640 medium supplemented with 10% fetal calf serum in 10% CO_2/90% air at 37°C. Twenty-four hours prior to assay, the cells are washed with serum-free medium and seeded in 24-well cluster trays.

        Assay of DNA Synthesis. Bombesin (1nM), 0.5µM [methyl-³H] thymidine (20 Ci/mmole, New England Nuclear), and four concentrations of the test compounds (1, 10, 100, 1000nM) are added to the cultures to achieve a final volume of 0.5 ml. After a 28 hr incubation at 37°C, the cells are collected onto GF/B glass fiber filters, and the DNA is precipitated with ice-cold TCA. [³H] thymidine incorporation into acid-insoluble fractions of DNA is determined by liquid scintillation spectrometry.

Phase 3 - Peptide-Induced Pancreatitis

        Male, Sprague-Dawley rats (250g) are used for these experiments. The test compound, or 0.9% NaCl is administered s.c. 15 min. prior to the bombesin

injection. Bombesin injections are given s.c. at a dose of 10 µg/kg, and blood samples are obtained at 1 hr.30 min., 3hr. and 6hr. Plasma amylase concentration are determined by the Pantrak Amylase test.

Phase 4- In Vitro Inhibition of [^{125}I] Gastrin Releasing Peptide (GRP) Binding to Bombesin Receptors

Membranes from various tissues (rat brain, rat pancreas, rat anterior pituitary, SCLC, 3T3 cells) are prepared by homogenization in 50mM TrisHCl containing 0.1% bovine serum albumin and 0.1mg/ml bacitracin followed by two centrifugations (39,000xgx15 min., 4°C) with an intermediate resuspension in fresh buffer. For assay, aliquots (0.8ml) are incubated with 0.5nM [^{125}I]GRP (~2000 Ci/mmol, Amersham Corp.) and various concentrations of the test compounds in a final volume of 0.5ml. After a 30 minute incubation at 4°C, the binding reaction is terminated by rapid filtration through Whatman GF/C filters that have been pre-soaked in 0.3% aqueous polethyleneimine to reduce the level of nonspecific binding. The filters and tubes are washed three times with 4ml aliquots of ice-cold buffer, and the radioactivity trapped on the filters is counted by gamma-spectrometry. Specific binding is defined as the total.[^{125}I]GRP bound minus that bound in the presence of 1000nM bombesin or a related peptide.

Phase 5- Inhibition of Gastrin Release

The stomachs of anesthetized rats are perfused with saline collected over 15 minute periods via pyloric cannulation while the test peptide is infused through the femoral vein for periods between 0 and 150 minutes.

Phase 6- In Vivo Antitumor Activity

NCI-H69 small cell lung carcinoma cells were transplanted from in vitro culture by implanting each animal with the equivalent of 5 confluent 75 cm^2 tissue culture flasks in the right flank. In vitro NCI-H69 cells grow as a suspension of cellular aggregates. Therefore, no attempt was made to disaggregate the cell agglomerates by physical or chemical means. Tumor size was calculated as the average of two diameters, i.e., (length and width/2) mm.

Phase 7 - Inhibition of Growth Hormone Release

Anterior pituitaries from adult male rats, weighing 200-250 g and housed under controlled conditions (lights on from 0500-1900 h), were dispersed using aseptic technique by a previously described trypsin/DNase method (Heiman et al., Endocrinology, 1985, 116, 410) derived from other methods (Hoefer et al., Mol. Cell. Endocrinol., 1984, 35, 229).

The dispersed cells were diluted with sterile-filtered Dulbecco's modified Eagle medium (MEM) (Gibco Laboratories, Grand Island, NY (GIBCO)), which was supplemented with fetal calf serum (2.5%; GIBCO), horse serum (3%; GIBCO), fresh rat serum (10% stored on ice for no longer than 1 h) from the pituitary donors, MEM nonessential amino acids (1%; GIBCO), gentamycin (10/mg/mL; Sigma) and nystatin (10,000 U/mL; GIBCO). The cells were counted with a hemacytometer (approximately 2,000,000 cells per pituitary) and randomly plated at a density of 200,000 per well (Co-star cluster 24; Rochester Scientific Co., Rochester, NY). The plated cells were maintained in the

above Dulbecco's medium in a humidified atmosphere of 95% air and 5% CO_2 at 37°C for 96 h.

In preparation for a hormone challenge, the cells were washed 3x with medium 199 (GIBCO) to remove old medium and floating cells. Each dose of secretagogue (diluted in siliconized test tubes) was tested in quadruplicate wells in medium 199 (total volume of 1 mL) containing BSA (1%; fraction V; Sigma Chemical Co., St. Louis, MO). Cells were pulsed in the presence of somatostatin (0.1 nM) to maintain control levels within narrow limits and to increase the ratio of maximally stimulated levels to basal secretory levels without adding additional growth factors or glucocorticoids. After 3 h at 37°C in an air/carbon dioxide atmosphere (95/5%), the medium was removed and stored at -20°C until assayed for hormone content.

Growth hormone (GH) in plasma and media was measured by a standard double antibody radioimmunoassay using components supplied by the NIDDK and the National Hormone and Pituitary Program, University of Maryland School of Medicine. Data are plotted as the mean values for a given dose of peptide obtained by pooling the means from individual experiments done in quadruplicate. The number of experiments for each analogue is given in Table 6. Potencies and 95% confidence intervals were calculateds by 4-point assay (Pugsley, Endocrinology, 1946, 39, 161).

Results of Assays of Test Peptides

A number of analogs of bombesin or GRP, each containing a non-peptide bond or a statine, AHPPA, or ACHPA, ß-amino acid, or Y-amino acid residue, can be synthesized and tested in one or more of the

above-described Phase 1 - 7 assays; the results of Phase
1 and 2 tests are given in Table 1 attached hereto.
Table 1 shows formulas for the non-peptide analogues and
results of $\underline{in}$ $\underline{vitro}$ inhibition of [^{125}I]GRP binding to
3T3 fibroblast bombesin receptors, and
bombesin-stimulated [^{3}H]Thymidine uptake by cultured
3T3 cells. (3T3 GRP receptor and thymidine uptake data
are expressed in IC50 (nM).) Table 1 also gives results
for non-peptide bond-containing analogs of one other
naturally-occurring peptide, Neuromedin C, whose
C-terminal seven amino acids are similar to those of
bombesin and GRP. (In Table 1, "Litorin" indicates a 9
residue peptide analog or its derivative, whereas
"Neuromedin C" indicates a 10 residue analog or its
derivative.)

In Table 1, the position of the non-peptide
bond is indicated by the position of the symbol
ψ[CH$_2$NH]; i.e., ψ[CH$_2$NH] is always shown
preceding the amino acid which, in that peptide, is
bonded to the amino acid N-terminal to it via the
non-peptide bond. Where no amino acid is specified
under "structure", the non-peptide bond links the two
peptides represented by the numbers given as
post-scripts.

In Table 1, it can be seen that a preferred
placement of the non-peptide bond in litorin analogs is
at the A^8 - A^9 position; two of the most active
analogs (as indicated by a low GRP receptor IC50 value)
are BIM-26100 (Phe^8ψ[CH$_2$NH]Leu^9) and BIM-26101
$Leu^8\psi[CH_2NH]Leu^9]$.

In addition, as shown in Table 1, BIM-26113
(D-Phe^1, Leu^8ψ[CH$_2$NH]Leu^9) and BIM-26114
(D-Nal^1, Leu^8ψ[CH$_2$NH]Leu^9) are active in the

3T3 GRP receptor binding and thymidine uptake assays.
Most notably, BIM-26136 (D-Nal^1,
Leu$^8\psi$[CH$_2$NH]Phe^9), which contains amino and
carboxy terminal aromatic residues that are capable of
5   forming a hydrophobic interaction, is the most potent
analog. Finally, when statine or ß-leucine replaces the
A^8 and A^9 residues of litorin, the resultant analogs
BIM-26120 and BIM-26182 are also potent antagonists.

        Table 1 also shows that Neuromedin C analogs
10   containing a non-peptide bond between residues A^9 -
A^{10}, e.g., BIM-26092, 26105, 26106, and 26107, are
antagonists when tested in the 3T3 GRP receptor and
thymidine uptake assays.

        Table 1 also gives negative results for analogs
15   of Neuromedin C and GRP 19-27, e.g., BIM-26108. Thus
the non-peptide bond placement guidelines given herein
should be used in conjunction with the routine assays
described above to select useful antagonists.

        Bombesin and Bombesin analogs have been shown
20   to inhibit the effect of interleukin-2 (IL-2) (Fink et
al., 1988, Klin. Wochenschr. 66, Suppl. 13, 273). Since
IL-2 causes T lymphocytes to proliferate, it is possible
that litorin antagonists may prevent the inhibitory
effect of Bombesin or its analogs on IL-2. IL-2
25   stimulated lymphocytes are capable of effectively lysing
small cell lung carcinoma cells in vitro. Although
Bombesin antagonists have a direct antiproliferative
effect on neoplastic tissues, they may also favor
proliferation of lymphocytes having lytic activity for
30   small cell lung carcinoma.

        These observations prompted us to evaluate the
effect of BIM-26100 on the in vivo growth of the SCLC
tumor cell line described in Phase 6. Twenty athymic

nude females, 5 to 6 weeks of age, were implanted on day
0 with the NCI-H69 human SCLC, individually identified
and then randomized into the following vehicle control
and test groups:

Group No.	Treatment	No. Animals
1	Saline vehicle treated control:	
	0.2 ml, s.c. inf., b.i.d., QD1-28	10
2	BIM-26100:	
	50ug/inj., s.c., b.i.d., QD1-28	5
3	BIM-26100:	
	50ug/inj., s.c. inf., b.i.d., QD1-28	5

(s.c. = subcutaneously; inf. = infused around tumor;
inj. = injected; b.i.d. = twice per day; QD1-28 = daily
treatment, on days 1 - 28.)

Growth of NCI-H69 xenografts and the tumor
growth inhibitory activity of the bombesin antagonist
BIM-26100
(pGlu-Gln-Trp-Ala-Val-Gly-His-Pheψ[CH$_2$NH]Leu-NH$_2$)
are illustrated as tumor growth curves in Fig. 1, and
relative tumor sizes in Table 2. Administration of
BIM-26100 as a s.c. infusion around the tumor
significantly inhibited tumor growth. The effectiveness
of the antitumor activity of BIM-26100 is evident in
view of the large inoculum of NCI-H69 tumor cells (i.e.,
the equivalent of 5 confluent 75 cm^2 cell culture .
flasks per animal) and the agglomerated condition of the
cells. In confluent flasks, NCI-H69 agglomerates are
macroscopically visible and together resemble a
metastatic tumor colony. Many such tumor colonies were
implanted per animal. The dose of BIM-26100 was
arbitrarily selected on the bases of compound
availability and is not optimal. Higher doses of
BIM-26100 may be administered, as indicated by body
weight gain (minus tumor weight) gain during the course
of treatment (Table 3). This suggest BIM-26100
completely lacks local or systemic toxicity and is

useful therapeutically as an anti-growth factor with
anti-tumor effects.

D-Phe-Gln-Trp-Ala-Val-N-methyl-D-Ala-His-Leu-
methylester and

D-F_5-Phe-Gln-Trp-Ala-Val-D-Ala-His-Leu-methylester
were examined for their abilities to displace
^{125}I-labeled bombesin from rat pancreatic acini cells
and to inhibit amylase release from these cells produced
by bombesin itself. The analogues exhibit potencies in
the half-maximal effective dose ranges of 5-10 nM and
are thus potent bombesin receptor antagonists.

Testing of GRF Analogs

The purified analogs were assayed in a 4-day
primary culture of male rat anterior pituitary cells for
growth hormone (GH) release, as described by Hocart et
al. (1988, supra) and Murphy and Coy (1988, Peptide
Research 1:36). Potential antagonists were retested in
the presence of GRF(1-29)NH_2 (1 nM). The results are
shown in Figs. 4-6, in which different dosages of the
analogs were measured against GRF.

The incorporation of the reduced peptide bond
isostere in the N-terminal region of GRF(1-29)NH_2
produced very weak agonists and one antagonist with an
IC_{50} of approximately 10μM.

Placement of the pseudopeptide bond between the
N-terminal 9th and 10th residues produced the analogue
[Ser^{9}ψ[CH$_2$NH]Tyr^{10}]- GRF(1-29)NH_2 (peptide
VIII). This analog was found to be inactive in the
potency assay, and was therefore tested for antagonist
activity in the presence of a stimulating dose of
GRF(1-29)NH_2 (1 nM). The results are shown
graphically in Fig. 6. [Ser^{9}ψ[CH$_2$NH]Tyr^{10}]-
GRF(1-29)NH_2 was found to be an antagonist in the
10μM range vs 1 nM GRF. Earlier conventional

structure-activity studies with the same peptide had elucidated a more potent antagonist, namely [N-Ac-Tyr^1, D-Arg^2]GRF(1-29)NH$_2$ (Robberecht et al., J. Endocrinology, 1985, 117, 1759). This analog had an IC$_{50}$ of approximately 1μM in an assay for adenylate cyclase activity in rat anterior pituitary homogenates.

Placement of the pseudopeptide bond in any one position between the remaining N-terminal 11 amino acids produced analogs having less activity than GRF(1-29)NH$_2$. These results are presented in Table 6 (n = number of separate experiments in quadruplicate from which the corresponding curves in the Figures were calculated). Incorporation of the isosteres Tyr^{1}ψ[CH$_2$NH]Ala^2 and Ala^{2}ψ[CH$_2$NH]Asp^3, gave weak agonists with ~0.1 activity of the control (peptide I, Tyr^{1}ψ [CH$_2$NH]Ala^2, 0.12%, and peptide II, Ala^{2}ψ[CH$_2$NH]Asp^3, 0.13%). At position 3, the isostere Asp^{3}ψ[CH$_2$NH]Ala^4 (peptide III) produced the most potent agonist of the series which retained 1.6% of the activity of the control. When Ala^{4}ψ[CH$_2$NH]Ile^5 was incorporated at position 4 (peptide IV), the activity dropped to 0.02% of GRF(1-29)NH$_2$. This drop continued at position 5, where the isostere Ile^{5}ψ[CH$_2$NH]Phe^6 (peptide V) produced the least active agonist with a potency of <0.01% of that of the control. Phe^{6}ψ[CH$_2$NH]Thr^7 (peptide VI) produced a potency to 0.13% of that of GRF(1-29)NH$_2$ but the isostere Thr^{7}ψ[CH$_2$NH]Asn^8 produced a weak agonist (peptide VII, 0.02% potency). With the isostere Ser^{9}ψ[CH$_2$NH]Tyr^{10} in the peptide (VIII), all trace of agonist activity was lost at doses as high as

0.1 mM. Another agonist was produced with $Tyr^{10}\psi[CH_2NH]Arg^{11}$ in the peptide although it too had low potency (IX, 0.39%).

The loss of potency observed after placement of the pseudopeptide bond at each position of the first 11 N-terminal positions of GRF(1-29) was greater than that seen with smaller peptides, such as somatostatin and bombesin which contain ß-bends in the region of the molecule important for receptor recognition. Chou-Fasman analysis shows GRF to be predominantly α-helical in character in the biologically important N-terminal portion of the molecule. The replacement of the naturally-occurring C=O bond by the $C=H_2$ bond of the pseudopeptide bond has pronounced effects on α-helical formation due to a loss of intramolecular H-bonding sites and increased rotational freedom about the isostere C-N bond. Loss of intermolecular H-bonding sites might also induce changes in receptor binding capabilities of both ß-bend and α-helical peptides. However, given the dramatic loss in activity of these GRF analogues, as compared to somatostatin and bombesin, effects on peptide conformation are probably more important in the larger helical GRF molecule.

Use

The peptides of the invention may be administered to a mammal, particularly a human, in one of the traditional modes (e.g., orally, parenterally, transdermally, or transmucosally), in a sustained release formulation using a biodegradable biocompatible polymer, or by on-site delivery (e.g., in the case of anti-cancer bombesin to the lungs) using micelles, gels and liposomes.

The bombesin antagonists of the invention are suitable for the treatment of all forms of cancer where bombesin-related substances act as autocrine or paracrine mitotic agents, particularly small-cell lung carcinoma. The peptides can also be used for the inhibition of gastric acid secretion and motility disorders of the GI tract, the symptomatic relief and/or treatment of exocrine pancreatic adenocarcinoma, and the restoration of appetite to cachexic patients. The peptides can be administered to a human patient in a dosage of 0.5 μg/kg/day to 5 mg/kg/day. For some forms of cancer, e.g., small cell lung carcinoma, the preferred dosage for curative treatment is 250mg/patient/day.

The compound can be administered to a mammal, e.g., a human, in the dosages used for growth hormone releasing factor or, because of their decreased potency, in larger dosages. The compounds can be administred to a mammal, e.g., a human, in a dosage of 0.01 to 1000 mcg/kg/day, preferably 0.1 to 100 mcg/kg/day.

<u>Table 1</u>

Code	Structure	3T3 GRP Receptor IC50(nM)	Thym. Uptake IC50(nM)
BIM-26092	Gly-Asn-His-Trp-Ala-Val-Gly- His-Leuψ[CH$_2$NH]Leu-NH$_2$ Neuromedin C	242	466
BIM-26095	pGlu-Gln-Trp-Ala-Val-D-Ala- His-Leuψ[CH$_2$NH]Leu-NH$_2$ Litorin	2623	1209
BIM-26100	pGlu-Gln-Trp-Ala-Val-Gly-His- Pheψ[CH$_2$NH]Leu-NH$_2$ Litorin	23	26
BIM-26101	pGlu-Gln-Trp-Ala-Val-Gly-His- Leuψ[CH$_2$NH]Leu-NH$_2$ Litorin	118	296
BIM-26105	D-Ala-Asn-His-Trp-Ala-Val- D-ALa-His-Leuψ[CH$_2$CH]Leu-NH$_2$ Neuromedin C	107	107
BIM-26106	desGly-D-Ala-His-Trp-Ala-Val- D-Ala-His-Leuψ[CH$_2$CH]Leu-NH$_2$ Neuromedin C	401	354
BIM-26107	D-Phe-His-Trp-Ala-Val-Gly- His-Leuψ[CH$_2$NH]Leu-NH$_2$ Neuromedin C	199	154

SEQ ID No: 11

Table 1 (cont'd)

Code	Structure	3T3 GRP Receptor IC50(nM)	Thym. Uptake IC50(nM)
BIM-26108	N-Ac-D-Ala-His-Trp-Ala-Val- Gly-His-Leuψ[CH$_2$NH]Leu-NH$_2$ GRP (19-27)	841	>1000
BIM-26113	D-Phe-Gln-Trp-Ala-Val-Gly- His-Leuψ[CH$_2$NH]Leu-NH$_2$ Litorin	5.8	9
BIM-26114	D-Nal-Gln-Trp-Ala-Val-Gly- His-Leuψ[CH$_2$NH]Leu-NH$_2$ Litorin	23.5	28
BIM-26120	pGlu-Gln-Trp-Ala-Val-Gly- His-Sta-NH$_2$ Litorin	150	165
BIM-26122	D-Phe-Gln-Trp-Ala-Val-Gly- His-Leu-NH$_2$ Litorin	5.9	28.6
BIM-26136	D-Nal-Gln-Trp-Ala-Val-Gly-His- Leuψ[CH$_2$NH]Phe-NH$_2$ Litorin	1.4	3.3
BIM-26182	D-Cpa-Gln-Trp-Ala-Val-Gly-His- ß-Leu-NH$_2$ Litorin	0.88	4.77

TABLE II

IN VIVO TUMOR INHIBITORY ACTIVITY OF THE BOMBESIN ANTAGONIST BIM-26100: NCI-H69 HUMAN SCLC

Group No.	Treatment	Tumor Size[1] Day 18 (mm)	% Test/Control	Tumor Size Day 28 (mm)	% Test/Control
1	Vehicle treated control, 0.2ml, s.c. inf., b.i.d. QD1-28	10.9±1.82		15.9±2.27	
2	BIM-26100, 50μg/inj., s.c., b.i.d., QD1-28	10.1±1.47	93	17.3±1.96	108
3	BIM-26100, 50μg/inj., s.c. inf., b.i.d. QD1-28	7.6±1.56**	70	13.7±0.67*	86

[1]Data reported as means ± SD on 10 animals in the control and 5 in test groups.
Student's t Test Significance of difference from control: *$p<0.05$; **$p<0.01$.

TABLE III

EFFECT OF TUMOR GROWTH AND BIM-26100 TREATMENT ON BODY WEIGHT: LACK OF SYSTEMIC TOXICITY

Group No.	Treatment	Body Weight(gm)[1] Day 0	Body Weight(gm) Day 18	Body Weight(gm) Day 28
1	Vehicle treated control, 0.2ml, s.c. inf., b.i.d., QD1-2	17.3	19.6	19.7
2.	BIM-26100, 50µg/inj., s.c., b.i.d., QD1-28	16.9	19.2	19.1
3	BIM-26100, 50µg/inj., s.c. inf. b.i.d., QD1-28	17.7	20.4	21.1

[1] Body weights are presented as means of 10 animals in the control and 5 in test groups: Tumor weights calculated from 2 largest diameters in mm converted to mgs using the formula for an ellipsoid (length x width 2/2)mgs. were subtracted from total body weights.

Table 4. Peptide Chromatographic, Purity and
Mass Spectral Data

Peptide	Analogue	HPLC t_R /min	Purity /%	FAB-MS (M-H+)
	GRF(1-29)NH$_2$	16.9	99.4	
I	Tyr$^1\psi$[CH$_2$NH]Ala^2	16.2	99.4	3345
II	Ala$^2\psi$[CH$_2$NH]Asp^3	14.9	94.1	3345
III	Asp$^3\psi$[CH$_2$NH]Ala^4	14.4	95.5	3345
IV	Ala$^4\psi$[CH$_2$NH]Ile^5	13.6	97.6	3345
V	Ile$^5\psi$[CH$_2$NH]Phe^6	14.3	97.2	3345
VI	Phe$^6\psi$[CH$_2$NH]Thr^7	14.7	95.3	33457
VII	Thr$^7\psi$[CH$_2$NH]Asn^8	14.7	96.7	3345
VIII	Ser$^9\psi$[CH$_2$NH]Tyr^{10}	15.8	92.6	3343
IX	Tyr$^{10}\psi$[CH$_2$NH]Arg^{11}	13.5	97.7	3344

Table 5. Amino Acid Analyses

	GRF-(1-29)NH$_2$	I	II	III	IV	V	VI	VII	VIII	IX
Asp	3.22	3.02	1.95	1.88	2.89	2.37	3.00	1.90	2.39	3.25
Thr	1.06	1.00	0.87	0.87	1.08	0.68			0.98	0.97
Ser	2.98	2.82	2.62	2.32	3.19	2.75	2.95	2.94	1.85	2.81
Glu	2.25	2.20	2.50	1.81	2.24	2.19	2.25	2.09	2.25	2.10
Gly	1.12	1.10	1.04	1.01	1.03	1.13	0.95	0.97	1.08	1.00
Ala	3.30	2.19	2.23	2.03	2.20	1.80	3.03	3.14	3.13	3.07
Val	0.75	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Met	0.99	1.13	1.00	0.91	0.98	1.00	1.01	0.96	0.89	0.97
Ile	1.77	1.87	1.83	1.59	1.08	1.06	1.11	1.57	1.90	1.88
Leu	4.00	4.00	3.75	2.92	4.12	4.04	4.05	3.38	4.08	4.06
Tyr	1.92	1.11	1.65	1.73	2.04	1.23	2.02	2.00	0.94	0.92
Phe	0.70	0.91	0.94	0.86	0.90			0.93	0.94	1.07
Lys	2.02	1.81	1.63	2.37	2.15	2.08	1.97	2.01	2.24	1.82
Arg	3.16	2.91	2.90	2.89	3.11	3.14	3.10	3.07	3.40	2.14

Table 6. In Vitro Biological Potencies of Reduced Peptide Bond GRF(1-29)NH$_2$ Analogues

Peptide	Analogue	Potency (%)	95% Confidence Interval	n
	GRF(1-29)NH$_2$	100	-	28
I	Tyr$^1\psi$[CH$_2$NH]Ala^2	0.12	0.07-0.22	7
II	Ala$^2\psi$[CH$_2$NH]Asp^3	0.13	0.08-0.24	7
III	Asp$^3\psi$[CH$_2$NH]Ala^4	1.6	0.9 -3.0	4
IV	Ala$^4\psi$[CH$_2$NH]Ile^5	0.02	0.01-0.05	4
V	Ile$^5\psi$[CH$_2$NH]Phe^6	<0.01	n.d.	7
VI	Phe$^6\psi$[CH$_2$NH]Thr^7	0.13	0.07-0.25	7
VII	Thr$^7\psi$[CH$_2$NH]Asn^8	0.02	0.01-0.03	4
VIII	Ser$^9\psi$[CH$_2$NH]Tyr^{10}	-	-	5
IX	Tyr$^{10}\psi$[CH$_2$NH]Arg^{11}	0.39	0.23-0.67	6

Other embodiments are within the following claims.

What is claimed is: